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14. ABSTRACT
Our objective is to create a multi-institutional tissue microarray resource from radical prostatectomy samples with detailed clinical information and follow-up and rigorous case-cohort design for use as a platform for validating tissue biomarkers of prognosis. In addition, we have proposed testing a series of biomarkers of prognosis and a set of biomarkers that correlate with Gleason Score. We have made significant progress over the past year. We have completed the tissue microarrays and finalized standard procedures for tissue microarray storage, sectioning and shipping. We have set up a structure for reviewing and approving biomarker proposals based on sound scientific principles and strong preliminary data. We have devised and tested a centralized distribution mechanism at Stanford University of collating and shipping TMAs to participating sites, We have found shortcomings with the BLISS system and STMAD for histological image capture and storage for pathological review and have developed a much improved, highly efficient system using a Leica scanner and PathXL image analysis software suite. We also have made significant progress in testing TACOMA, an automater TMA scoring algorithm. We have completed staining of the TMAs for H & E, High Molecular Weight Keratin, p27, ERG, SPKINK1, Ki67 (MIB1), MUC1, Survivin and PTEN FISH. Over the next year we will carry out more staining, complete refinements of the infrastructure, complete pathologic review of these and other biomarkers, evaluate concordance of scoring of immunohistochemical staining amongst study pathologists and report (publish) our findings.

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ABSTRACT

Our objective is to create a multi-institutional tissue microarray resource from radical prostatectomy samples with detailed clinical information and follow-up and rigorous case-cohort design for use as a platform for validating tissue biomarkers of prognosis. In addition, we have proposed testing a series of biomarkers of prognosis and a set of biomarkers that correlate with Gleason Score. We have made significant progress over the past year. We have completed the tissue microarrays and finalized standard procedures for tissue microarray storage, sectioning and shipping. We have set up a structure for reviewing and approving biomarker proposals based on sound scientific principles and strong preliminary data. We have devised and tested a centralized distribution mechanism at Stanford University of collating and shipping TMAs to participating sites, We have found shortcomings with the BLISS system and STMAD for histological image capture and storage for pathological review and have developed a much improved, highly efficient system using a Leica scanner and PathXL image analysis software suite. We also have made significant progress in testing TACOMA, an automater TMA scoring algorithm. We have completed staining of the TMAs for H & E, High Molecular Weight Keratin, p27, ERG, SPKINK1, Ki67 (MIB1), MUC1, Survivin and PTEN FISH. Over the next year we will carry out more staining, complete refinements of the infrastructure, complete pathologic review of these and other biomarkers, evaluate concordance of scoring of immunohistochemical staining amongst study pathologists and report (publish) our findings.

Introduction

Intense debate over the utility of prostate cancer screening has been sparked by four large randomized trials reported over the past few years. Two European trials have shown benefits to diagnosis and treatment of prostate cancer, namely, the Scandinavian Prostate Cancer Study Group 4 randomized trial of surgery vs. watchful waiting for localized prostate cancer and the European Randomized Study of Screening for Prostate Cancer. However, two North American trials, PLCO and PIVOT, have shown no mortality benefit to screening and treatment for prostate cancer. While these studies have engendered a lively debate among researchers and urologists, they have also sewn confusion among patients and practitioners regarding the best course when faced with an elevated PSA or diagnosis of localized prostate cancer. One possible interpretation of these studies is that earnest application of systematic PSA screening in the North American population over the past 20 years has led to a profound shift in grade, stage, and tumor volume that has changed the biology of prostate cancer to predominantly indolent disease. PSA has been a victim of its own success. Regardless of the outcome of the debate, these studies collectively testify to significant over-diagnosis and over-treatment of prostate and argue strongly for increased use of active surveillance (AS), particularly in men with low risk disease. Unfortunately, AS remains underutilized as an approach because of uncertainties regarding the true biologic potential of most diagnosed tumor. In the face of uncertainty, the vast majority of patients and physicians opt for treatment and too often suffer the consequences.

We began our multi-institutional Canary Tissue Microarray Project as a direct response to the need for validated biomarkers of prognosis. A search of the terms prognosis, biomarker, and

prostate cancer will retrieve literally hundreds of candidates, yet none are in routine use clinically. This problem is not unique to prostate cancer, but plagues the biomarker field broadly. We have used rigorous clinical trial case/cohort design, taking care to correct for institutional and spectrum biases (see attached review). Despite support from the Canary Foundation, insufficient resources had significantly slowed our progress in building this resource. Funding from the Department of Defense allowed us to complete construction of necessary infrastructure and begin testing biomarker candidates. We are pleased to report our progress after 1 year.

Specific Aim 1) To test markers of prognosis on prostate cancer tissue microarrays with associated clinical data.

1.A. Develop work-flow for TMA sharing, image scanning, TMA staining data analysis.

We are pleased to report that, as promised, the multi-institutional TMAs have been constructed at all sites. A total of 495 patients with recurrence after surgery and 621 patients without recurrence have been included in the final microarrays. Patients have been selected at random from the pool of patients who had undergone radical prostatectomy at each of the sites, with special attention to selecting patients with features typical of low-intermediate risk patients seen in contemporary urologic practices. Details of patient selection, statistical considerations, and TMA construction are summarized in an attached manuscript which has been submitted recently (Appendix 1). In addition to this cohort, a separate TMA has been constructed from patients 220 who underwent radical prostatectomy at a sister site who have very long term follow-up (up to 25 years) and hard endpoints including metastases and prostate cancer specific death. Since many of these patients were diagnosed in the pre-and early PSA eras, they are held separately as a validation cohort.

We have completed several stated aims in the proposal with regard to development of work-flow for array sharing, analysis and archiving while some aspects continue to be developed:

1) After TMA manufacture was completed, Standard Operating Procedures (SOPs) have been developed at each site for TMA storage, sectioning and transferal. All TMA blocks are stored under nitrogen at each site and as proposals for biomarkers are reviewed and approved 10 sections are cut each time. Since storage of cut sections can lead to degradation of some epitopes, we have chosen to section when proposals are approved and reagents ready.

2) We have developed an infrastructure for shipping slides from each participating site to a central site (Stanford University). There, the slides are sorted so that a complete set (for the 1100 patients which are spread over 32 separate slides) of slides is collated. These complete sets are then shipped to the site where they will be stained. Thus far we have shipped sets to the University of Washington and Queen's University in Canada. We have also successfully shipped slides from Stanford University, and the University of Washington to the University of British Columbia (UBC) for image capture (see below).

3) We have completed H & E staining of the complete set at Stanford University. IN addition, we have stained the complete set for high molecular weight keratins to aid the pathologists in interpreting slides (cancers are HMWK negative, while normal glands show staining in the basal cells of glands). The H & E and HMWK stained slides have been shipped to UBC for scanning and image archiving for use by all pathologists.

4) Image capture and archiving is currently under development and nearly completed. We had proposed using the BLISS system for TMA core image capture with the intent to use the Stanford Tissue MicroArray Database (STMAD) for storing images and making them available as digital images for the pathologists to view and score. While STMAD is powerful, it has some limitations in functionality that the pathologists did not like, including a lack of flexibility in scoring parameters for biomarkers. Furthermore, BLISS required nearly a full day to scan a single TMA slide at high resolution. Since our cohort is spread over 32 slides, it would take over a month of scanning to acquire images for a single biomarker, which is obviously unacceptable. UBC has acquired a Leica SCN400 Slide Scanner with the SL801 Autoloader and this is available to this project at no cost by our collaborators. The Leica scanner can capture high resolution images of an entire TMA in about 1 hour and is fully automated so a deck of slides can be loaded and scanned. The images generated are automatically ported into the __ image analysis software suite. This system has the advantage of flexibility in setting up scoring parameters and image manipulation that STMAD lacked. The pathologists are delighted with the improvement and are currently scoring the first of our markers.

5) Data management: Sites transmitted all of their clinical data to the DMCC under Dr. Feng's direction for selection of cases for the TMA as detailed in our proposal. Now that the TMAs have been constructed, the sites have sent complete, HIPAA compliant clinical information for all patients included on the TMA for their site. These data have now been centralized in their final form in the DMCC and are summarized in the accompanying table (Supporting Data; Table 1). The clinical data are therefore complete and ready for correlation to the biomarker staining data from the TMAs.

6) Automated tissue image scoring: We have tested and refined our automated scoring algorithm, TACOMA (Tissue Array Co-Occurance Matrix Analysis) [REF], in preparation for this study's patient samples. This computer-based scoring will accelerate our evaluation of potential markers by eliminating the need for every pathologist to score hundreds of TMA images from each site. By triaging poorly-performing markers we can focus resources on the most promising markers. Our algorithm has now been successfully tested on a CD117 marker using 1050 images in the STMAD and achieved performance sufficient for the goals of the study---a success rate ~85% is, in fact, conservative based on consultation with study pathologists who reviewed "misclassified" samples. As a part of this pilot study, we also devised a pre-filter based on hue which allows us to flexibly focus the algorithm toward tissue staining properties deemed most important by pathologists. Additional algorithmic training and refinement is underway using TMA images from several prostate cancer studies (lead by Drs. Janet Stanford and Lawrence True). We are collaborating with the UBC investigators to coordinate all efforts

related to the logistics of their new scanner and software for both the computer-based marker prioritization and pathologists' expert evaluation.

1.B. Test candidate biomarkers of prognosis for prediction of recurrence after radical prostatectomy

In monthly conference calls, the TMA investigators review progress and review applications for utilizing the TMAP resource. Most applications for use of the TMAs come from within the group, although it is available to the prostate cancer research community broadly and can be accessed by application through the Canary Foundation website (http://www.canaryfoundation.org). We have focused on biomarkers that have well characterized, highly performing reagents (e.g. immunohistochemical grade antibodies) and sufficient preliminary data that they could supply prognostic information independent of grade, stage and PSA. We have begun staining for biomarkers listed in our proposal.

1) Proposed biomarkers: We have completed immunohistochemical staining for ERG, SPINK1, Survivin, p27 (KIP1) and Ki67 (MIB1). In all cases, the staining was at exceptionally high quality per initial review of the glass slides by our pathologists. Slides have been shipped to UBC and are currently being scanned. For our initial biomarker, ERG, 3 or more pathologists will read all 1100 cores and score them so we can investigate reproducibility of scoring. Scores will be correlated with clinical outcome. Since our TMA is uniquely designed for high level validation of markers, we intend to publish finding whether positive or negative so that poorly performing biomarkers can be discarded. In addition to immunohistochemistry, we have shipped slides to Jeremy Squire at Queens hospital for FISH to interrogate copy number alterations (allelic loss) at the PTEN locus.

2) Other biomarkers are in the pipeline for staining including MUC1, EZH2 and digital image analysis of H & E stained slides that has been found to be highly prognostic in breast cancer. Over the next year we will be expanding our portfolio of biomarkers. By accumulating data on many biomarkers on the same cohort we will be positioned to investigate whether combinations of biomarkers improve prediction. For instance, we will be able to investigated whether PTEN and ERG status predict failure after surgery, as has been reported previously.

Specific Aim 2) To evaluate candidate markers that correlate with Gleason grade on prostate cancer tissue microarrays with associated clinical data.

Thus far, we have focused on building the analysis pipeline and in staining high priority biomarkers of prognosis. The intent of this aim is to investigate biomarkers that correlate with Gleason grade. Several markers are in our queue and are listed in the original proposal. For some, we are still looking for high quality affinity reagents that provide interpretable staining with limited background. Leading candidates are AGR2, a marker expressed at high levels in Gleason pattern 3 cancers and Monoamine oxidase A, expressed at high levels in Gleason pattern 4 disease.

For all biomarkers, whether for Gleason score or prognosis, the statistical analysis strategy has been outlined in our proposal and will be used as soon as reads are available from the pathologists.

Key Research Accomplishments

- Completion of construction of TMAs at all participating sites
- Standardizing and deploying Standard Operating Procedures for TMA storage, sectioning and shipping at each site
- Centralized shipping, collation and distribution of TMAs at Stanford University
- Biomarker review and approval by the investigative team to ensure quality of the reagents and sufficient level of evidence for investigation of a particular biomarker on our valuable resource.
- Inclusion of investigators (Dr. Squire) in the broad prostate cancer research community for testing candidate biomarkers. The resource has been proven to be available to the community broadly.
- Porting final clinical data that will be used for analysis of biomarker performance to the University of Washington DMCC.
- Deployment of a more efficient image capture system (Leica) so that we can increase the throughput of biomarker testing.
- Customization and use of a new image archiving and displaying software for management and scoring of the immunohistochemical staining by the study pathologists
- Completion of foundational staining for H & E and HMWK that will be used by the pathologists in the interpretation of all immunohistochemical stains of the TMA. In addition, importing of these stains into the image database as common resource.
- Completion of staining for ERG, SPINK1, Survivin, p27 (KIP1) and Ki67 (MIB1). We anticipate publishing separate manuscripts for Survivin, ERG and SPINK, p27, and Ki67.
- Development of TACOMA algorithm that has potential for automated TMS scoring reducing pathologist reading time and facilitate objective evaluation of biomarkers.

Reportable Outcomes

1) Publication of a review of the challenges to biomarker development in a high impact journal by Dr. Brooks:

James D. Brooks: Translational genomics: The challenge of developing cancer diagnostic biomarkers. *Genome Research* **22:** 183-187, 2012.

2) Submission of a review article on the creation of the TMA resource. This manuscript serves as a template for investigators working in any disease for design and creation of a multi-institutional TMA resource as we have done. The submitted manuscript is attached in the **Appendix** and is noted to be protected as unpublished.

Sarah Hawley, Ladan Fazli, Jesse K. McKenney, Jeff Simko, Dean Troyer, Marlo Nicolas, Lisa F. Newcomb, Janet E. Cowan, Luis Crouch, Michelle Ferrari, Javier Hernandez, Antonio

Hurtado-Coll, Kyle Kuchinsky, Janet Liew, Rosario Mendez-Meza, Elizabeth Smith, Imelda Tenggarra, Xiaotun Zhang, Peter R. Carroll, June M. Chan, Martin Gleave, Raymond Lance, Daniel W. Lin, Peter S. Nelson, Ian M. Thompson, ***Ziding Feng***, Lawrence D. True and ***James D. Brooks***: Design and construction of a resource for the validation of candidate prognostic biomarkers: the Canary Prostate Cancer Tissue Microarray as a model. Manuscript submitted, 2012.

3) Publication of TACOMA method in a high impact journal by the co-investigator of this grant Dr. Randolph:

D. Yan, P. Wang, M. Linden, B. Knudsen, ***T. Randolph***, "Statistical methods for analyzing tissue images---algorithmic scoring and co-training", Annals of Applied Statistics, 2012, Vol. 6, No. 3, 1280–1305.

Conclusion

We have undertaken a challenging task of creating a multi-institutional TMA resource with rigorous case/cohort design. To our knowledge, such a resource has not been previously created and offers the advantage of reducing institutional biases as well as spectrum biases. In the uniform design and through image acquisition and archiving technologies, we have created a resource that can be easily used by the greater prostate cancer research community. In many ways, this resource represents a gold standard by for evaluation of prognostic biomarkers. We are delighted and excited to report completion of nearly all phases of pipeline construction and that we have begun analysis of biomarkers on this resource. We anticipate rapid progress in analyzing multiple prognostic biomarkers over the next year as well as important publications. This research directly addresses the PCRP overarching challenge to *distinguish lethal from indolent disease*.

Supporting Data

Table 1: Summary of study participants by recurrence status, follow-up time after radical prostatectomy, Gleason score and site.

	Stanford	UCSF	UW	UBC	UT	EVMS	Total
Recurrent Patients	88 (18%)	101 (20%)	102 (21%)	20 (4%)	81 (16%)	103 (21%)	495
<5 yrs. FU post-RP	72	91	92	14	71	92	432
<=6	19	25	19	1	14	48	126
3+4	27	52	27	7	24	33	170
4+3	35	10	35	3	6	6	95
8-10	11	4	11	1	25	5	57
UNK	0	0	0	2	2	0	4
>5 yrs. FU post-RP	16	10	10	6	10	11	63
<=6	2	3	3	3	6	3	20
3+4	9	5	5	1	2	5	27
4+3	4	3	1	1	1	1	11
8-10	1	0	1	1	1	2	6

	Stanford	UCSF	UW	UBC	UT	EVMS	Total
Non-recurrent Patients	84 (14%)	98 (16%)	106 (17%)	100 (16%)	127 (20%)	106 (17%)	621
<5 yrs. FU post-RP	13	9	10	19	24	12	87
<=6	1	4	4	7	15	11	42
3+4	9	4	2	11	6	1	33
4+3	2	1	1	1	2	0	7
8-10	1	0	3	0	1	0	5
>5 yrs. FU post-RP	71	89	96	81	103	94	534
<=6	22	40	52	50	55	59	278
3+4	37	41	37	22	30	32	199
4+3	9	7	6	5	6	3	36
8-10	3	1	1	4	12	0	21

RP=radical prostatectomy

FU=follow-up